Controlled whey protein hydrolysis using two alternative proteases

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Abstract

Whole whey was hydrolyzed for 12 h with Protease 2A and Trypsin using two concentrations of enzyme (20 and 40 g/kg$_{protein}$). Samples were assayed for total viable counts of adventitious microflora that survived thermization, total acidity, total concentration of free amino acids, peptide profile and overall degree of hydrolysis. The highest total concentration of free amino acids was observed when hydrolysis was effected by Protease 2A, and the major variations in amino acid qualitative composition occurred between 2 and 6 h: Leu exhibited the most significant increase, followed by Lys, Phe and Ile. Hydrolysis with Trypsin led to release of high amounts of Lys. Quantitative depletion of β-lactoglobulin was observed by 2 h under all processing conditions, and hydrolysis of α-lactalbumin was slower when Trypsin was employed. Formation of peptides was more extensive under the action of Trypsin than of Protease 2A, and the major peptides released by the former had molecular weights mainly in the ranges 7500–8000 and 4000–4500 Da, whereas those released by the latter accumulated in the range 7000–7500 Da. The differences between the hydrolytic actions of Trypsin and Protease 2A were significant except with respect to the concentration of Glu, as well as degree of breakdown of immunoglobulin G and β-lactoglobulin. Growth of adventitious bacteria and generation of free amino acids were successfully modeled using postulated mathematical models. The values of v_{max} for Trypsin were 0.15 and 0.06 g/(l h) for 40 and 20 g/kg$_{protein}$, respectively, and for Protease 2A were 0.86 and 0.50 g/(l h) for 40 and 20 g/kg$_{protein}$, respectively.

Keywords: Dairy; Trypsin; Enzyme; Protein; Proteolysis

1. Introduction

Cheese whey is a strong pollutant owing to its high content in lactose and protein; although downstream processing of this liquid effluent may generate considerable amounts of proteins as solid material, only about one half of them are used as ingredients for human food or animal feed (Kosaric & Asher, 1985), where they may serve as supplement, or partial substitute of other proteins. It has been well established that partial enzymatic hydrolysis of proteins reduces their antigenicity, so immune responses triggered by their peptide length are decreased (Asselin, Amiot, Gauthier, Mourad & Hebert, 1988; Asselin, Hebert & Amiot, 1989; Nakamura, Sado & Sykunobe, 1993a; Nakamura, Sado, Sykunobe & Hirota, 1993b; Tossavainen, Outinen, Harju & Makinen-Kiljunen, 1996). Hydrolysis of whey proteins affects also their functional properties, and may even enhance several important rheological characteristics (Kuehler & Stine, 1974; Gauthier, Paquin, Poulliot & Turgeon, 1993; Sato, Imai, Nakamura, Nishiya, Kawanari & Nakajima, 1996a, b). A wide range of fine chemicals can be obtained from whey fermentations, including alcohols, organic acids, vitamins and biopolymers (Yang & Silva, 1995). However, whey is not always an economically feasible feedstock for industrial fermentations because it is low in available organic nitrogen as required for biomass build-up (Christopherson & Zottola, 1989; Mulligan, Safi & Groleau, 1991; Proulx, Gauthier & Roy, 1992; Proulx, Ward, Gauthier & Roy, 1994; St-Gelais, Roy, Haché & Desjardins, 1993). Most whey fermentations do in fact use supplementation with yeast extract to achieve acceptable rates of growth and productivity (Aeschlimann & Von Stockar, 1989; Chiarini, Mara & Tabacchioni, 1992). However, controlled hydrolysis of proteins in whole whey may provide a nitrogen source suitable for growth promotion, hence eliminating (or reducing) the need for expensive supplements (Perea, Ugalde, Rodriguez & Serra, 1993). On the other hand, hydrolysis of whey facilitates its sterilization; owing to the high thermal sensitivity of whey proteins, they precipitate easily during heat treatment which can be prevented by preliminary cleavage of

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peptide bonds (Briand, Chobert & Haertle, 1994). This research effort has therefore tried to deepen the characterization of hydrolyses of skimmed whole whey using two types of commercial enzymes (Trypsin and Protease 2A), in the presence of controlled microbial growth (as pasteurization rather than sterilization was applied to the whey), with the final goal of producing a self-enriched medium suitable for fermentation.

2. Materials and methods

2.1. Feedstock and chemicals

Blue dextran, aldolase and bovine serum albumin were obtained from Pharmacia (Uppsala, Sweden). NaCl, NaOH, KH_2PO_4, K_2HPO_4, HCl, KOH, $HClO_4$, 5-sulphosalicilic acid, formaldehyde, lactose, N-Leu and silicone were purchased from Merck (Darmstadt, Germany). Tryptic Soy Agar (TSA) was purchased from Lab M (Bury, UK). Peptone was obtained from Oxoid (Basingstoke, UK). Protease 2A, an enzyme produced by *Aspergillus niger* with high proteolytic activity, was obtained from Amano Pharmaceutical (Nagoya, Japan); Trypsin from bovine pancreas (T-8253) was purchased from Sigma (St. Louis MO, USA). Bovine whey was recovered as a by-product from the bulk manufacture of cheese from the milk of a native Portuguese bovine breed (*Cachena*). Bovine β-lactoglobulin, bovine α-lactalbumin, insulin chain B, insulin chain A and NAD were purchased from Sigma.

2.2. Processing and analytical equipment

Hydrolyses were carried out in a Braun Biostat B (Munchen, Germany), a 2-L fermentor with controls of pH, liquid level, temperature and surface foam. Total nitrogen was determined using the Microkjeltec system from Tecator (Höganäs, Sweden). Quantification of free amino acids was carried out using an automatic amino acid analyzer (Pharmacia). The peptide profile was determined in an FPLC system (Pharmacia) using a Superose 12 column, also from Pharmacia. Lactose and lactic acid were jointly quantified by HPLC (Merk) using an Aminex HPX-87X column (BioRad, Richmond CA, USA). Filters (0.22 μm nominal pore diameter) were purchased from Nucleopore (Cambridge MA, USA).

2.3. Enzymatic hydrolysis of whey

Plain bovine whey was completely skimmed prior to use. Each experiment used 1.0 l of whey pasteurized for 15 min at 62°C (to severely reduce the viability, and hence the enzymatic activity, of the adventitious microflora). The enzyme, Protease 2A or Trypsin, was then added at the ratio of 40 (or 20) g of enzyme protein per kg of soluble substrate protein, and the whole process was conducted at optimal performance conditions for both enzymes (i.e. pH 7 and 45°C for Protease 2A, and pH 8 and 37°C for Trypsin). The pH of each set of experiments was kept constant by automatic addition of alkali throughout hydrolysis. Two of these four experiments (Protease 2A added at the ratio of 40 g/kg and Trypsin at 20 g/kg) were replicated. The process was conducted under stirring at 300 rpm. and samples were collected aseptically every 2 h for a 12 h-period. After assessing microbial contamination in an aliquot, the remaining portion of the sample was immediately frozen for later physicochemical analyses.

2.4. Assay for microbial growth

As whole whey was previously submitted to pasteurization, only thermo-resistant microorganisms could grow in the medium during hydrolysis; in addition, aeration was provided at all times. In view of these two assumptions, assessment of microbial growth in whey during hydrolysis was done by total viable counts (colony forming units, cfu) in samples decimally diluted in sterile 1% (w/v) peptone water (Oxoid, Basingstoke, UK) and plated on Tryptic Soy Agar (TSA) (Lab M, Bury, UK) using the method of Miles & Misra (1938); plates were incubated aerobically for 24 h at 37°C. All analyses were carried out in duplicate.

2.5. Assay for chemical composition

The nitrogen content of whey was determined according to the IDF method (FIL/IDF 20B: 1993) adapted to micro conditions by using one tenth of all samples and reagents. Assays for free amino acids were carried out in an automatic amino acid analyzer (Pharmacia, Uppsala, Sweden) using sodium citrate buffer (Pharmacia) as eluant, an ion exchange column for separation and UV absorbance of ninhydrin adducts for detection. Prior to analysis, samples were pretreated as follows: to 1 ml of sample, 50 mg of solid 5-sulphosalycilic acid (Merck, Darmstadt, Germany) was added, the mixture was allowed to stand for 1 h at 4°C, centrifuged for 10 min at 4000 g and the supernatant solution added with enough 0.3 M lithium hydroxide (Pharmacia) to adjust pH to ca. 2; an internal standard, N-Leu (Sigma), was then added to the deproteinized liquid sample at the ratio 1:1 (v/v), and the mixture was finally filtered through a 0.22 μm membrane.

The molecular weight profile of peptides was estimated using an FPLC system (Pharmacia) equipped with a Superose-12 column (Pharmacia); separation conditions employed were similar to those used by Pintado & Malcata (1996) to separate major whey proteins.

The void volume of the column was determined using blue dextran. The column was calibrated for molecular weight using an aqueous solution containing 5.8 mg/ml of aldolase (MW: 158,000 Da), 7.0 mg/ml of bovine serum albumin (MW: 67,000 Da), 4.5 mg/ml of bovine β-lactoglobulin (MW: 36,000 Da), 2.0 mg/ml of bovine α-lactalbumin (MW: 14,000 Da), 2.5 mg/ml of insulin chain B (MW: 5750 Da), 2.5 mg/ml of insulin chain A (MW: 2531 Da) and 2.0 mg/ml of NAD (MW: 669 Da) as markers all from sigma.

The degree of hydrolysis (DH) throughout the reaction was indirectly evaluated by measuring the amount of α-amino nitrogen via the formaldehyde titration method (Wolfschoon & Vargas, 1978); the α-amino nitrogen reacts with formaldehyde, hence releasing H^+ cations, which are later quantified by potentiometric titration with NaOH to pH 9. The total content of peptide bonds used for calculation of DH was 8.32 equivalent per kg of protein (Adler-Nielssen, 1979). All analyses were carried out in duplicate. Lactose and lactic acid were quantified by HPLC; separation conditions were flow rate of 0.6 ml/min with 5 mM H_2SO_4 as eluant, sample injection volume of 50 μl, separation temperature of 60°C and detection by refractive index at 30°C for the disaccharide and UV absorbance for the organic acid. Prior to analysis, samples were pretreated in order to eliminate protein interference: 1 ml of sample was added with 100 μl of 35% (v/v) perchloric acid (PCA) to promote precipitation, allowed to stand for 10 min in ice, added with 55 μl of KOH 7.0 M to neutralize PCA, centrifuged for 10 min at 4000 g, and the supernatant filtered through a 0.22 μm membrane filter.

2.6. Statistical analysis

Analysis of variance was used to ascertain the effect of the manipulated variables (viz. type of enzyme, concentration of enzyme and time of incubation) on concentration of each free amino acid (and total free amino acids), concentration of peptides, microbial viable counts, total acidity and DH. The manipulated variables were tested in a twofold manner: (i) all data generated with Protease 2A or Trypsin were analyzed simultaneously, in order to determine the effect of the type of enzyme; and (ii) data generated with Protease 2A and with Trypsin were analyzed independently, in order to determine the effect of concentration of enzyme and time of hydrolysis in each case. Fisher's protected least significant difference *t*-test, at the 5% significance level, was applied in a pairwise fashion to all experimental results in order to assess statistically significant differences. All statistical analyses were implemented with the Statview™ package (Abacus Concepts, Berkeley CA, USA).

2.7. Mathematical analysis

Analyses of the experimental data suggested that growth of thermo-resistant adventitious microorganisms was affected to some extent by the concentrations of free amino acids, and that a small part of the free amino acid inventory produced during hydrolysis was consumed by these microorganisms. Therefore, the microbial growth in whey was assumed (i) to follow Monod growth kinetics and (ii) to depend on two limiting substrates, lactose and free amino acids, according to:

$$\frac{dC_X}{dt} = \mu_{max}\left(\frac{C_A}{K_A + C_A}\right)\left(\frac{C_L}{K_L + C_L}\right)C_X, \quad t \geqslant 0$$
$$C_X(t=0) = C_{X,0}, \tag{1}$$

where C_X is the concentration of viable microorganisms in whey at time t, C_A the concentration of free amino acids, C_L the concentration of lactose, μ_{max} is the maximum growth rate, K_A and K_L the Monod constants associated with free amino acid and lactose consumption, respectively, and subscript 0 denotes initial conditions.

In attempts to model the formation of free amino acids from whey protein by enzymatic hydrolysis, it was assumed that (i) the rate of enzyme-mediated production follows Michaelis–Menten kinetics on the concentration of substrate and that (ii) the microbial depletion of total free amino acids is growth-associated, viz:

$$\frac{dC_A}{dt} = \frac{v_{max}C_P}{K_m + C_P} - \frac{1}{Y_L}\frac{dC_X}{dt}, \quad t \geqslant 0,$$
$$C_A(t=0) = C_{A,0}, \tag{2}$$

where C_P is the concentration of protein after hydrolysis for time t, v_{max} the maximum reaction rate, K_m the Michaelis–Menten constant and Y_L the yield of biomass on lactose associated with substrate protein.

In attempts to model the lactose consumption, it was assumed that the total concentration of lactose, in view of its limiting status, is linearly related with biomass via the yield Y_L, viz.

$$C_L = \left(C_{L,0} + \frac{C_{X,0}}{Y_L}\right) - \frac{C_X}{Y_L}. \tag{3}$$

The initial content of lactose was 43.1 g/l.

In attempts to model the protein consumption, it was assumed that the total concentration of free amino acids is that of those amino acids originated by whey protein hydrolysis, which are variable depending on the type and concentration of enzyme (so consumption or release of free amino acids brought about by adventitious bacteria was taken as negligible), viz:

$$C_P = C_{P,0} - \alpha(C_A - C_{A,0}), \tag{4}$$

where α is a constant. The initial content of protein was 8.3 g/l. Although it may be claimed that the aforementioned assumption holds only if the enzymes were both

exoproteases or both unspecific, likelihood tests of the model form postulated did not unfold unjustified statistical bias, so there is no apparent reason to doubt the form of the model.

Since the biomass data are expressed in cfu/ml and the other parameters are expressed in g/l, supplementary conversion factors had to be introduced to avoid inconsistencies; Eq. (1) can thus to be rewritten as:

$$\frac{dC_X^*}{dt} = \mu_{max}\left(\frac{C_A^*}{K_A^* + C_A^*}\right)\left(\frac{C_L^*}{K_L^* + C_L^*}\right)C_X^*, \quad t \geqslant 0, \qquad C_X^*(t=0) = C_{X,0}^*, \tag{5}$$

where the dimensionless variables are defined as

$$C_X^* \equiv \frac{C_X}{C_{X,F}}, \tag{6}$$

where $C_{X,F}$ is the value of viable microorganisms by 12 h (the highest value ever attained) and $C_{X,0}$ was virtually nil,

$$C_L^* \equiv \frac{C_L}{C_{L,0}} \tag{7}$$

and

$$C_A^* \equiv \frac{C_A}{C_{A,0}}, \tag{8}$$

and where the dimensionless (adjustable) parameters are defined as

$$K_L^* \equiv \frac{K_L}{C_{L,0}} \tag{9}$$

and

$$K_A^* \equiv \frac{K_A}{C_{L,0}}. \tag{10}$$

By a similar token, Eq. (2) can be rewritten as

$$\frac{dC_A^*}{dt} = \frac{v_{max}^* C_P^*}{K_m^* + C_P^*} - \frac{1}{Y_L^*}\frac{dC_X^*}{dt}, \quad t \geqslant 0, \qquad C_A^*(t=0) = C_{A,0}^*, \tag{11}$$

where the new dimensionless variable is defined as

$$C_P^* \equiv \frac{C_P}{C_{P,0}} \tag{12}$$

and the new dimensionless (adjustable) parameters are defined by

$$v_{max}^* \equiv \frac{v_{max}}{C_{A,0}}, \tag{13}$$

$$K_m^* \equiv \frac{K_m}{C_{P,0}} \tag{14}$$

and

$$Y_A^* \equiv Y_A \frac{C_{A,0}}{C_{X,F}}. \tag{15}$$

The dimensionless counterpart of Eq. (3) then reads

$$C_L^* = \left(1 + \frac{C_{X,0}}{Y_L^*}\right) - \frac{C_{X,F}C_X^*}{Y_L^*}, \tag{16}$$

where the new dimensionless (adjustable) parameter is defined as

$$Y_L^* \equiv Y_L C_{L,0}. \tag{17}$$

The dimensionless counterpart of Eq. (4) reads

$$C_P^* = 1 - \alpha^*(C_A^* - 1), \tag{18}$$

where the new dimensionless (adjustable) parameter is defined by

$$\alpha^* \equiv \frac{\alpha\, C_{A,0}}{C_{P,0}}. \tag{19}$$

Eqs. (5), (11), (16), and (18) were integrated by finite differences in the process of estimating the adjustable parameters by nonlinear regression analysis to the data, using as objective function the minimization of the sum of squares of residuals.

3. Results and discussion

3.1. Growth of adventitious microflora

Whey obtained from cheesemaking with pasteurized milk contains significant amounts of microorganisms contributed by starters, coupled with contamination throughout processing. Growth of such microflora simultaneously with hydrolysis brought about by extraneous proteases may deplete small amounts of soluble proteins, thus decreasing the global inventory of substrate for posterior fermentation. Conversely, plain sterilization of whey leads to protein precipitation. Therefore, in order to inhibit microbial growth while keeping soluble proteins intact, whey was subject to thermization; initial viable numbers in whey, i.e. $(1.2 \pm 0.3) \times 10^5$ cfu/ml, were virtually reduced to zero after heat treatment (see Fig. 1a–d). The microbial groups that can survive that treatment are thus thermoresistant aerobes. As time elapses, growth of residual thermophilic microflora takes place gradually: with Trypsin at the ratio of 40 g/kg, low numbers of bacteria are present by 2 h (see Fig. 1c), while for Trypsin at the ratio of 20 g/kg, and for Protease 2A at both concentrations, a similar effect is observed not before 4 h (see Fig. 1a–c). These results are somewhat unexpected because incubation with Trypsin was done at 37°C; hence, the lower content in free amino acids when incubation was performed with this protease may have played a growth-limiting role. These results are relevant in selecting an incubation period that permits good hydrolysis yields without interference of microbial growth, which would be 2 h for Trypsin and 4 h for Protease 2A.

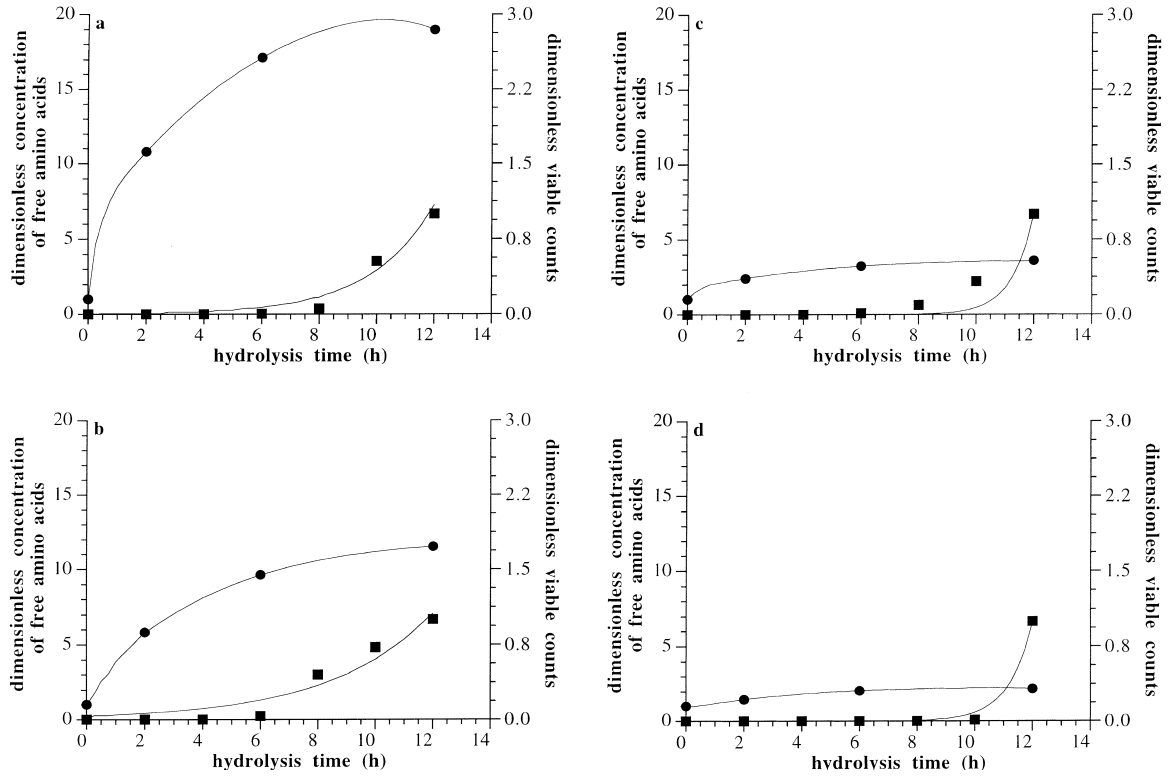

Fig 1. Growth data obtained for adventitious microflora (■), C_X^*, and total concentration of free amino acids (●), C_A^*, during incubation of whey with (a) Protease 2A at the ratio of 40 g and (b) 20 g of enzyme, both per kg of soluble substrate protein, (c) Trypsin at the ratio of 40 g and (d) 20 g of enzyme, both per kg of soluble substrate protein, and corresponding theoretical fits (____).

Data collected on total acidity (see Fig. 2) (based on the amount of NaOH required for maintenance of pH-stat conditions) indicated that Trypsin and Protease 2A are characterized by two well defined behaviors; these differences may be explained by the higher amounts of α-carboxylic groups made available by Protease 2A, as a non-specific enzyme which naturally cleaves more peptide bonds than Trypsin (which is specific for bonds involving basic amino acids, mainly Lys). Furthermore, the differences observed by 6 h can be accounted for by microbial metabolism, which exists at different levels by this time (see Fig. 1).

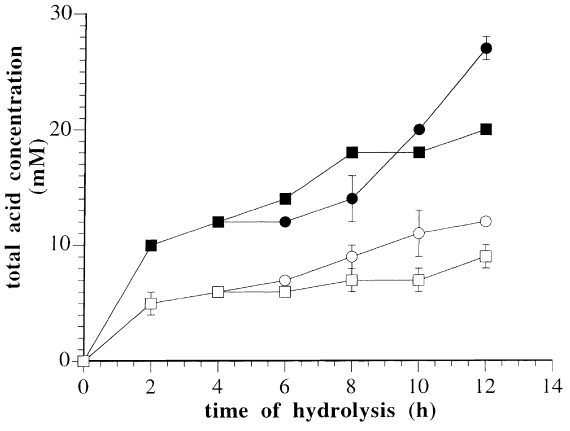

Fig 2. Acidification data, under pH-stat conditions, during incubation of whey with Protease 2A at the ratio of 40 g (●) and 20 g (■) of enzyme, both per kg of soluble substrate protein, and with Trypsin at the ratio of 40 g (○) and 20 g (□) of enzyme, both per kg of soluble substrate protein.

Formation of precipitates driven by heat treatment of whey hydrolyzates was observed when heating was at 90°C for 15 min: whey at pH 7.0 (which was used for hydrolysis by Protease 2A) produced a bulky precipitate, whereas whey at pH 8.0 (which was used for hydrolysis by Trypsin) led to little differences in turbidity before and after heat treatment. After hydrolysis for 2 h, whey treated with Protease 2A showed almost negligible precipitation, whereas after 4 h no precipitate was observed at all, which confirms disappearance of major whey proteins (α-La and β-Lg). Tryptic hydrolyzates did not undergo precipitation throughout time, owing probably to the higher pH used.

3.2. Amino acid composition of hydrolyzates

The total concentration of free amino acids (see Fig. 1) indicated significant differences between the two enzymes in terms of catalytic performance. The highest total free amino acid concentration was associated with the action of Protease 2A added at the higher ratio, followed by the same enzyme at the lower ratio; Trypsin

at the higher ratio came next, and Trypsin at the lower ratio came last. The main variations in free amino acid composition occurred between 0 and 6 h; although after this period only slight variations were observed, that does not necessarily indicate loss of enzymatic activity because, at this stage, microorganisms do actively consume free amino acids at rates sufficiently high to balance the former phenomenon.

The hydrolysis effected by Protease 2A (see Table 1), at either concentration, led to increases in the concentration of all free amino acids, a fact probably resulting from the unspecificity of that enzyme and from its high proteolytic activity; concentration of Leu exhibited the most significant increase, followed by Lys, Phe and Ile, to a lesser extent. In the first 2 h, Protease 2A at the ratio 40 g/kg caused the highest rate of release of all amino acids except Asp, Gly and Ala; Trp was only formed after 2 h. Between 6 and 12 h the concentrations of all free amino acids remained approximately constant except Thr, Ile, Leu and Lys; when the lower ratio of enzyme addition was used, the concentration of most free amino acids increased only slightly, except Leu, Lys, Phe, Ile and Val. After 6 h, Asn was not formed at all, whereas Trp was actively produced.

Hydrolysis brought about by Trypsin, at both levels, produced high amounts of Lys and slight formation of Arg, His and Leu, with essentially no effect on the other free amino acids; this observation may again be rationalized by the preference of this enzyme for peptide bonds involving basic amino acids (e.g. Lys). An approximately linear correlation was observed between the concentration of Trypsin used and that of Lys released. The free amino acid that has shown the smallest change in concentration throughout hydrolysis under all processing conditions was Glu, probably because the peptide bonds attacked by these enzymes do not liberate this amino acid (which exists as residue form at relatively high numbers). The decreases in concentration of a few free amino acids by 12 h, especially in the case of hydrolyzates produced via Protease 2A, could be justified by the fact that bacteria growing in whey can use free amino acids as nutrient for growth.

3.3. Peptide profile of hydrolyzates

The profile observed in unhydrolyzed whey (see Fig. 3a) is similar to that displayed elsewhere (Pintado & Malcata, 1996), with important peaks accounted for by α-La and β-Lg (major proteins of whey) and a further peak corresponding to orotic acid. After a period of incubation with enzyme, the major whey proteins are depleted and concomitantly a (not well resolved) group of peaks appears owing to peptide formation (see Figs. 3b–c). Peak integration of gel permeation chromatograms was duly performed; in order to simplify interpretation, the peaks detected in each chromatogram were lumped in arbitrary molecular weight fractions (see Table 2). The disappearance of the major whey proteins gives rise to generation of small peptides ranging between 500 and 8000 Da; this MW range is close to that reported by Nakamura et al. (1993a,b), viz. 300–10,000 Da for hydrolysis of whey protein concentrates carried out with proteases. The peaks associated with IgG and BSA did not show significant variation throughout hydrolysis, thus denoting constrained attack by these enzymes; this is possibly a consequence of their small concentration in whey. Analysis of Table 2 unfolds an almost complete disappearance of β-Lg by 2 h under all processing conditions, with no important differences in the corresponding rates of hydrolysis. Hydrolysis of α-La was slower at lower concentration of enzymes (as expected), and lower for Trypsin than for Protease 2A (see Table 2); this substrate protein vanished by 2 h with Protease 2A and by 6 h with Trypsin. In this particular, our results agree with those reported by Schmidt & Poll (1991), viz. α-La is slowly and partially hydrolyzed by Trypsin, and those by Antila (1989) who also claimed that α-La was the milk protein most resistant to hydrolysis by trypsin. A slower decrease in the concentration of α-La than β-Lg was also observed by Nakamura et al. (1993a,b) when a protease was employed.

The formation of peptides effected by the two enzymes was not essentially different from one another, but the dominant peptides produced by Trypsin exhibited MW mainly in the range 7500–8000 and 4000–4500 Da, and to a lesser extent in the range 1500–1000 Da, whereas those produced by Protease 2A appeared mainly in the 7000–7500 Da range and, to a lesser extent, in the 3500–3000 and 1000–8000 ranges. The fractions 2000–2500 Da and <800 Da are common outcomes of the action by both enzymes. In plain whey, the fractions 800–600 and <500 Da are essentially composed of orotic and uric acids, respectively, and new peaks, corresponding to small peptides, appear and become superimposed on such fractions as the degree of hydrolysis increases (thus increasing their lumped area). The major rate of formation of peptides by both enzymes occurs by ca. 2 h of incubation.

It is important to stress that the quantitative variation when going from one concentration of enzyme to another did not apparently lead to similar variation in peptide formation and free amino acid generation. The decrease in concentration of very small peptides in the last period of hydrolysis can be explained, as for free amino acids, by microbiological consumption.

3.4. Degree of hydrolysis of hydrolyzates

The DH, defined in our case as the percent of peptide bonds cleaved by either of the two enzymes tested, is a descriptor of the specific activity of each enzyme. The

Table 1
Concentration (mg/l) of free amino acids throughout incubation[a]

Amino acid	0 h	2 h				6 h			12 h				
	$PT_m \pm \sigma$	$P_{40} \pm \sigma$	P_{20}	T_{40}	$T_{20} \pm \sigma$	$P_{40} \pm \sigma$	P_{20}	T_{40}	$T_{20} \pm \sigma$	$P_{40} \pm \sigma$	P_{20}	T_{40}	$T_{20} \pm \sigma$
Asp	5.4 ± 1.0	8.2 ± 2.1	3.8	6.5	5.2 ± 0.4	13.6 ± 1.0	4.2	6.2	5.2 ± 0.8	12.6 ± 0.7	9.4	6.4	4.3 ± 0.9
Thr	1.2 ± 0.8	45.6 ± 4.0	26.0	3.1	1.9 ± 0.3	56.8 ± 2.0	49.0	3.7	2.0 ± 1.0	78.4 ± 6.4	57.6	3.6	1.2 ± 0.1
Ser	1.6 ± 1.3	16.6 ± 0.8	10.2	0.0	0.5 ± 0.5	22.9 ± 1.0	18.3	0.0	1.9 ± 1.9	23.4 ± 1.0	17.2	0.0	0.6 ± 0.6
Asn	0.0 ± 0.0	7.5 ± 4.2	0.0	0.0	0.0 ± 0.0	63.2 ± 1.5	0.0	0.0	0.0 ± 0.0	61.9 ± 3.2	0.0	0.0	0.0 ± 0.0
Glu	38.7 ± 6.9	42.4 ± 2.0	34.9	45.6	39.8 ± 6.4	45.5 ± 1.5	47.4	51.4	39.1 ± 3.7	33.2 ± 3.3	47.6	40.6	36.8 ± 4.0
Gln	0.0 ± 0.0	24.7 ± 0.1	24.7	0.0	0.0 ± 0.0	35.0 ± 1.1	19.8	0.0	0.0 ± 0.0	20.3 ± 0.8	17.0	0.0	0.0 ± 0.0
Gly	12.4 ± 2.8	15.5 ± 1.0	17.0	11.8	8.7 ± 0.0	24.0 ± 2.5	17.3	12.4	9.1 ± 0.0	22.3 ± 3.6	13.2	11.5	8.4 ± 0.0
Ala	3.9 ± 0.6	33.6 ± 4.3	5.9	4.0	2.6 ± 0.0	63.5 ± 2.6	5.9	4.9	3.0 ± 0.0	60.3 ± 5.1	3.8	3.8	2.7 ± 0.0
Val	2.6 ± 1.1	55.6 ± 0.5	31.5	2.9	2.2 ± 1.4	96.5 ± 0.8	62.0	2.8	4.9 ± 2.1	101.0 ± 8.4	80.6	3.8	4.9 ± 2.1
Met	0.0 ± 0.0	57.5 ± 0.6	46.5	0.0	0.0 ± 0.0	64.6 ± 1.8	58.6	0.0	0.0 ± 0.0	69.5 ± 0.3	61.0	0.0	0.0 ± 0.0
Ile	0.2 ± 0.4	60.7 ± 2.7	38.5	0.0	0.0 ± 0.0	109.0 ± 0.5	66.7	0.0	0.0 ± 0.0	135.2 ± 7.5	80.5	5.0	0.0 ± 0.0
Leu	5.1 ± 0.2	151.8 ± 0.3	94.2	3.8	1.0 ± 1.0	238.5 ± 5.0	162.3	12.5	5.3 ± 0.9	290.0 ± 8.8	205.1	37.7	10.7 ± 2.4
Tyr	0.3 ± 0.6	39.4 ± 0.1	30.8	0.0	0.0 ± 0.0	56.5 ± 1.5	31.3	0.0	0.0 ± 0.0	65.1 ± 5.6	43.0	1.9	0.5 ± 0.0
Phe	0.9 ± 0.9	71.4 ± 1.9	12.3	0.0	0.0 ± 0.0	113.1 ± 2.2	71.7	6.0	2.5 ± 2.5	128.3 ± 7.6	96.8	9.0	2.5 ± 2.5
Orn	1.2 ± 0.7	4.8 ± 0.6	7.2	5.2	2.4 ± 0.8	8.0 ± 1.5	4.3	10.1	6.6 ± 1.8	19.3 ± 2.3	4.5	4.8	5.2 ± 3.8
Lys	7.8 ± 1.2	102.9 ± 4.7	63.2	106.5	59.7 ± 1.9	156.2 ± 2.7	103.1	130.3	89.3 ± 6.6	186.0 ± 1.3	123.6	159.5	90.6 ± 5.7
His	0.3 ± 0.7	35.8 ± 0.4	19.9	0.0	0.6 ± 0.6	55.6 ± 4.0	48.6	3.0	3.0 ± 3.0	56.4 ± 1.9	44.0	13.6	0.5 ± 0.5
Try	0.0 ± 0.0	0.0 ± 0.0	0.0	0.0	0.0 ± 0.0	21.5 ± 1.4	0.0	0.0	0.0 ± 0.0	25.0 ± 0.3	22.7	0.0	0.0 ± 0.0
Arg	3.7 ± 1.3	57.5 ± 2.3	38.2	15.6	13.8 ± 2.2	76.3 ± 1.6	62.0	21.5	15.3 ± 1.7	72.2 ± 8.6	68.7	4.6	20.2 ± 4.4

[a] PT_m: mean of data for all conditions tested; P_{40}: Protease 2A at 40g/$kg_{protein}$; P_{20}: Protease 2A at 20g/$kg_{protein}$; T_{40}: Trypsin at 40g/$kg_{protein}$; T_{20}: Trypsin at 40g/$kg_{protein}$; σ: standard deviation.

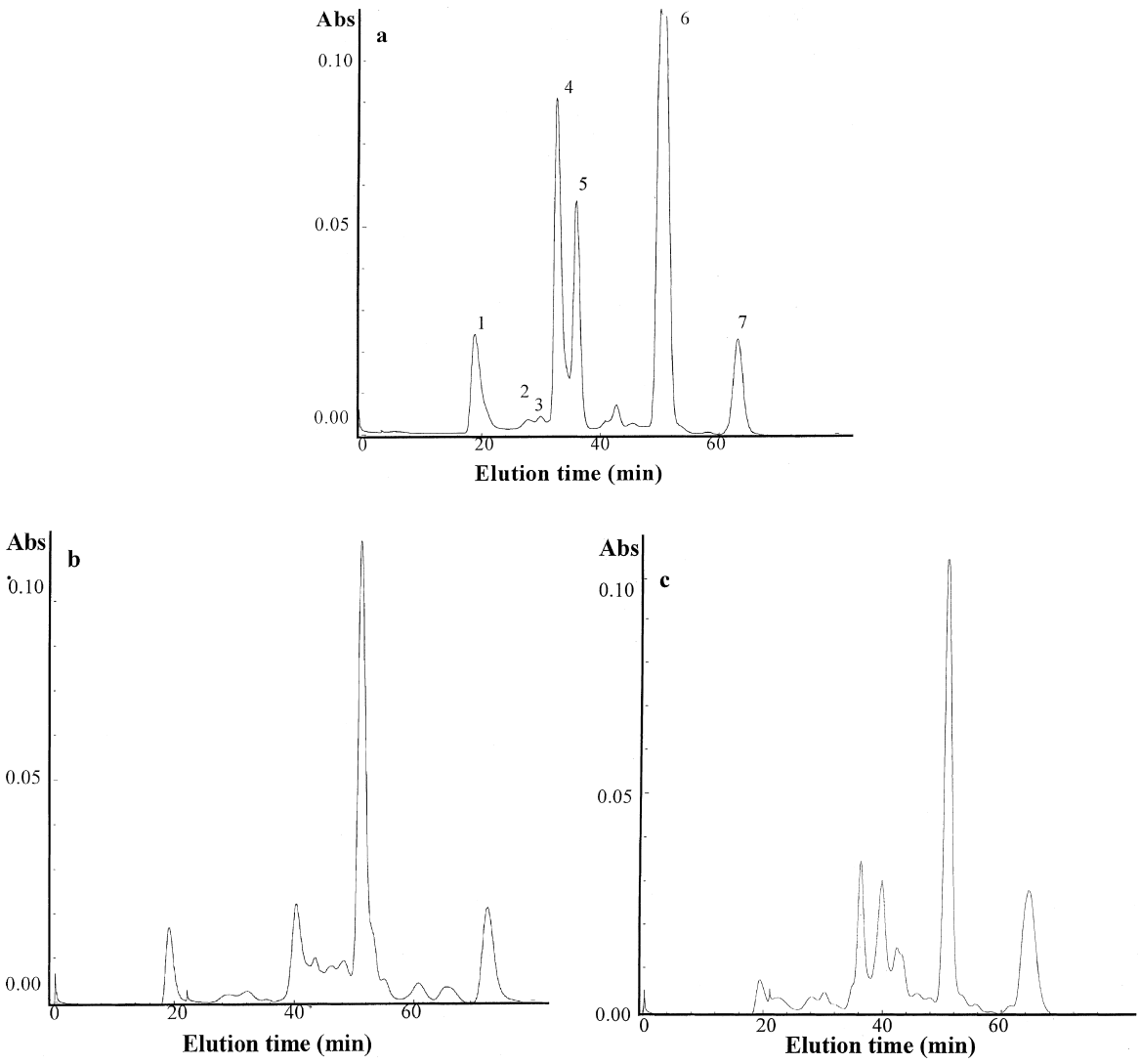

Fig 3. FPLC profile of (a) plain whey and whey after hydrolysis for 12 h (b) with protease 2A and (c) with Trypsin, both at the ratio of 40 g of enzyme per kg of soluble substrate protein. 1: aggregates, 2: immunoglobulin G, 3: bovine serum albumin, 4: β-lactoglobulin, 5: α-lactalbumin, 6: orotic acid, 7: uric acid.

values associated with digestion by Trypsin (see Fig. 4) are of the same order of magnitude of those obtained by Sato et al. (1996a,b) and Asselin et al. (1989), whereas the values associated with digestion by Protease 2A (see Fig 5) are in ranges similar to those reported by Iturbe-Chiñas and Canales (1988). The DH is highest for the higher concentration of Protease 2A (23%) and lowest for the lower concentration of Trypsin (7%). The degree of hydrolysis by Trypsin tended to level off by 2 h, and the increase in DH with time for Protease 2A was more notorious than that for Trypsin.

3.5. Statistical analysis

The analysis of variance performed on all data obtained following hydrolysis by Trypsin and Protease 2A suggested that a significant difference ($p < 0.050$) existed between these enzymes in terms of the concentrations of all free amino acids but Glu ($p = 0.750$), concentration of total free amino acids, total viable counts, total acidity, DH, and amounts of all peptide fractions except IgG ($p = 0.612$) and β-Lg ($p = 0.076$); these results are consistent with trends of the experimental data discussed above. Analysis of the effects of concentration of enzyme and time of hydrolysis, for each enzyme considered per se, have emphasized the existence of significant differences (see Tables 3 and 4). For Protease 2A, significant differences existed between the upper and lower level in terms of concentrations of all free amino acids except Asp ($p = 0.054$), Thr ($p = 0.077$), Ser ($p = 0.062$), Glu ($p = 0.333$), Gly ($p = 0.055$) and Arg ($p = 0.084$), as well as for total acidity ($p = 0.238$). The differences in the enzyme level did not cause significant differences in any peptide fractions except for α-La, 3000–3500 Da, 1500–

Table 2
Peak area (AUxtime) of peptide ranges and proteins throughout incubation[a]

MW Fraction (Da)	0 h	2 h				6 h				12 h			
	$PT_m \pm \sigma$	$P_{40} \pm \sigma$	P_{20}	T_{40}	$T_{20} \pm \sigma$	$P_{40} \pm \sigma$	P_{20}	T_{40}	$T_{20} \pm \sigma$	$P_{40} \pm \sigma$	P_{20}	T_{40}	$T_{20} \pm \sigma$
150.000 (IgG)	13.2 ± 1.6	9.8 ± 0.9	8.6	8.0	9.1 ± 0.5	8.8 ± 0.9	8.3	7.0	8.5 ± 1.3	6.4 ± 0.0	6.6	6.9	8.7 ± 2.8
67.000 (BSA)	8.0 ± 0.8	13.4 ± 0.9	8.6	9.2	11.0 ± 0.8	7.2 ± 0.9	11.1	15.2	11.5 ± 1.1	8.5 ± 0.0	6.7	11.8	12.5 ± 1.8
36.000 (β-Lg)	124.2 ± 6.5	5.1 ± 0.4	5.6	3.2	11.8 ± 1.9	0.0 ± 0.0	0.0	0.0	0.0 ± 0.0	0.0 ± 0.0	0.0	0.0	0.0 ± 0.0
14.400 (α-La)	79.8 ± 3.2	8.6 ± 1.0	29.9	48.8	55.1 ± 5.4	0.0 ± 0.0	0.0	10.9	27.0 ± 1.2	0.0 ± 0.0	0.0	0.0	0.0 ± 0.0
7.500–8.000	0.0 ± 0.0	0.0 ± 0.0	0.0	55.8	62.7 ± 1.5	0.0 ± 0.0	0.0	63.6	58.7 ± 2.5	0.0 ± 0.0	0.0	61.1	61.8 ± 0.1
7.000–7.500	0.0 ± 0.0	57.1 ± 0.1	54.3	0.0	0.0 ± 0.0	59.0 ± 2.0	54.7	0.0	0.0 ± 0.0	54.5 ± 1.0	53.4	0.0	0.0 ± 0.0
4.000–4.500	0.0 ± 0.0	0.0 ± 0.0	0.0	45.0	41.8 ± 2.2	0.0 ± 0.0	0.0	56.9	43.8 ± 3.7	0.0 ± 0.0	0.0	54.7	42.0 ± 3.0
3.000–3.500	13.1 ± 1.0	22.9 ± 1.1	35.3	0.0	0.0 ± 0.0	15.3 ± 1.7	23.2	0.0	0.0 ± 0.0	22.6 ± 2.4	20.7	0.0	0.0 ± 0.0
1.500–2.000	4.5 ± 0.1	13.2 ± 0.3	12.5	12.0	11.5 ± 0.1	8.9 ± 2.1	19.4	14.4	10.6 ± 0.5	15.9 ± 0.3	22.0	13.8	11.4 ± 0.0
1.000–1.500	0.0 ± 0.0	0.0 ± 0.0	0.0	10.4	9.9 ± 0.9	0.0 ± 0.0	0.0	17.2	10.9 ± 1.0	0.0 ± 0.0	0.0	0.0	3.5 ± 3.5
800–1.000	0.0 ± 0.0	16.9 ± 0.2	13.5	0.0	0.0 ± 0.0	20.3 ± 0.8	22.3	0.0	0.0 ± 0.0	20.2 ± 1.6	19.7	0.0	0.0 ± 0.0
600–800	156.9 ± 5.7	180.6 ± 3.9	175.7	144.8	163.1 ± 0.2	184.0 ± 2.8	189.3	170.4	162.6 ± 9.7	183.4 ± 0.6	144.1	169.5	162.4 ± 7.2
<500	46.8 ± 3.4	102.8 ± 2.0	102.4	98.5	67.1 ± 3.8	86.9 ± 1.4	98.4	89.8	64.7 ± 0.0	85.3 ± 2.4	55.5	66.7	77.4 ± 7.5

[a] PT_m: mean of data for all conditions tested; P_{40}: Protease 2A at 40g/kg$_{protein}$; P_{20}: Protease 2A at 20g/kg$_{protein}$; T_{40}: Trypsin at 40g/kg$_{protein}$; T_{20}: Trypsin at 40g/kg$_{protein}$; σ: standard deviation. IgG: Immunoglobulin G; BSA: Bovine Serum Albumin; α-La: α-lactalbumin; β-Lg: β-lactoglobulin; AU: absorbance units.

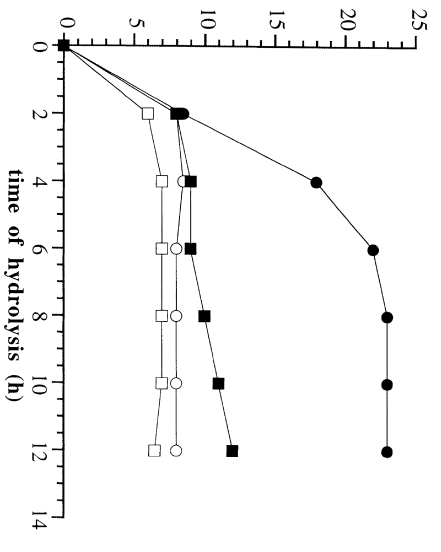

Fig 4. Evolution of degree of hydrolysis throughout incubation time of whey with Protease 2A at the ratio of 40 g (●) and 20 g (■) of enzyme, both per kg of soluble substrate protein, and with Trypsin at the ratio of 40 g (○) and 20 g (□) of enzyme, both per kg of soluble substrate protein.

2000 Da, 1000–1500 Da, 1000–800 Da and 600–800 Da. The differences between times of hydrolysis are more significant at initial than at later stages. In what concerns Trypsin, the differences between the two levels of enzyme were not significant with regard to the concentrations of all free amino acids but Leu and Lys, and for all peptide fractions except those corresponding to 4000–4500 Da, 1500–2000 and <500 Da, as well as for α-La, for total viable counts and for DH; similar results were obtained with Protease 2A, although differences were more significant at initial times than later in the incubation period.

3.6. Mathematical analysis

The theoretical fits using the best estimates of parameters are overlaid on the experimental data in Fig. 1. The estimates of the parameters obtained are tabulated in Table 6. Inspection of Fig. 1 indicates that in general the fits are good with respect to formation of free amino acids, and reasonable for viable numbers of the (uncontrolled) microflora.

Estimates tabulated in Table 5 indicate that K_A is very low and possibly not functionally significant in the model within the experimental range tested. The value of μ_{max} is well estimated for both enzymes; the value obtained for whey hydrolyzed by Trypsin is higher when the temperature of incubation is 37°C. When the concentration of Protease 2A changes from 20 to 40 g/kg, the value of μ_{max} becomes approximately double as expected, but the same did not unexpectedly happen for Trypsin. The values of K_L and Y_L are acceptable from a physicochemical point of view, and remain approximately constant for each enzyme. As anticipated, when the concentrations of both enzymes change from 20 to

Table 3
Fisher's protected least significant difference *t*-test applied pairwise to concentrations of individual free amino acids and total free amino acids (TAA)[a]

Source of variation		Asp	Thr	Ser	Asn	Glu	Gln	Gly	Ala	Val	Met	Ile	Leu	Tyr	Phe	Orn	Lys	His	Try	Arg	TAA
Protease 2A																					
C	20–40	0.052	0.077	0.062	0.002*	0.333	0.001*	0.055	0.000*	0.000*	0.002*	0.001*	0.000*	0.005	0.001*	0.032*	0.000*	0.010*	0.000*	0.084	0.000*
T	0–2	0.322	0.005*	0.002*	0.019*	0.029*	0.000*	0.504	0.007	0.000*	0.000*	0.000*	0.000*	0.000*	0.000*	0.069	0.000*	0.000*	–	0.000*	0.000*
	0–6	0.082	0.003*	0.001*	0.001*	0.006*	0.000*	0.022*	0.001*	0.000*	0.000*	0.000*	0.000*	0.000*	0.000*	0.034*	0.000*	0.000*	0.000*	0.000*	0.000*
	0–12	0.023*	0.001*	0.001*	0.000*	0.018*	0.000*	0.013*	0.001*	0.000*	0.000*	0.000*	0.000*	0.000*	0.000*	0.002*	0.000*	0.000*	0.000*	0.000*	0.000*
	2–6	0.226	0.106	0.032*	0.001*	0.041*	0.002*	0.043*	0.006*	0.000*	0.001*	0.001*	0.000*	0.028	0.001*	0.524	0.000*	0.001*	0.000*	0.017*	0.000*
	2–12	0.052	0.008*	0.014*	0.000*	0.501	0.002*	0.024*	0.010*	0.000*	0.000*	0.000*	0.000*	0.003*	0.000*	0.007*	0.000*	0.001*	0.000*	0.018*	0.000*
	6–12	0.290	0.040*	0.448	0.022*	0.071	0.000*	0.585	0.582	0.005*	0.022*	0.007*	0.002*	0.045	0.017*	0.012*	0.001*	0.729	0.000*	0.935	0.001*
Trypsin																					
C	20–40	0.194	0.059	0.269	–	0.382	–	0.788	0.161	0.495	–	–	0.002*	–	–	0.513	0.000*	–	–	0.411	0.000*
T	0–2	0.770	0.599	0.521	–	0.935	–	0.975	0.957	0.823	–	–	0.153	–	–	0.406	0.000*	–	–	0.029*	0.000*
	0–6	0.682	0.360	0.983	–	0.735	–	0.751	0.506	0.453	–	–	0.123	–	–	0.054	0.000*	–	–	0.012*	0.000*
	0–12	0.414	0.924	0.530	–	0.565	–	0.615	0.975	0.366	–	–	0.001*	–	–	0.186	0.000*	–	–	0.024*	0.000*
	2–6	0.904	0.667	0.534	–	0.796	–	0.775	0.538	0.345	–	–	0.021	–	–	0.152	0.006*	–	–	0.373	0.002*
	2–12	0.582	0.664	0.988	–	0.618	–	0.636	0.933	0.277	–	–	0.000*	–	–	0.541	0.002*	–	–	0.847	0.000*
	6–12	0.663	0.404	0.543	–	0.805	–	0.867	0.488	0.860	–	–	0.002*	–	–	0.333	0.115	–	–	0.471	0.054

[a] C: concentration of enzyme (mg/kg$_{protein}$), T: time of hydrolysis (h).
* Significant at the 5% significance level.

Table 4
Fisher's protected least significant difference *t*-test at the 5% significance level applied pairwise to lumped peak areas of peptide fractions, counts of viable bacteria (cfu), total acidity (TAc) and degree of hydrolysis (DH)[a]

Source of variation		IgG	BSA	β-Lg	α-La	7500–8000	7000–7500	4000–4500	3000–3500	1500–2000	1000–1500	800–1000	600–800	<500	cfu	TAc	DH
Protease 2A																	
C	20–40	0.270	0.561	0.061	0.000*	–	0.249	–	0.038	0.015	–	0.000*	0.004*	0.000*	0.000*	0.2377	0.000*
T	0–2	0.023	0.015	0.000*	0.000*	–	0.000*	–	0.002*	0.002*	-	0.000*	0.001*	0.000*	–	0.000*	0.000*
	0–6	0.017	0.595	0.000*	0.000*	–	0.000*	–	0.088	0.003*	–	0.000*	0.038*	0.000*	0.927	0.000*	0.000*
	0–12	0.004*	0.921	0.000*	0.000*	–	0.000*	–	0.012	0.001*	–	0.000*	0.837	0.004*	0.000*	0.000*	0.000*
	2–6	0.722	0.024	0.032	0.000*	–	0.954	–	0.009	0.662	–	0.007*	0.037*	0.000*	0.927	0.030*	0.000*
	2–12	0.070	0.013	0.032	0.000*	–	0.967	–	0.059	0.020	–	0.015*	0.000*	0.000*	0.000*	0.000*	0.000*
	6–12	0.105	0.532	–	–	–	0.921	–	0.108	0.013	–	0.413	0.006*	0.000*	0.000*	0.000*	0.150
Trypsin																	
C	20–40	0.958	0.362	0.210	0.000*	0.632	–	0.029	0.762	0.006*	0.961	–	0.871	0.002*	0.003*	0.028	0.250
T	0–2	0.013	0.158	0.000*	0.000*	0.000*	–	0.000*	0.000*	0.000*	0.002*	–	0.366	0.000*	0.999	0.000*	0.000*
	0–6	0.007	0.024	0.000*	0.000*	0.000*	–	0.000*	0.000*	0.000*	0.001*	–	0.178	0.000*	0.945	0.000*	0.000*
	0–12	0.007	0.34	0.000*	0.000*	0.000*	–	0.000*	0.000*	0.000*	0.040	–	0.193	0.000*	0.000*	0.000*	0.000*
	2–6	0.450	0.149	0.000*	0.000*	0.985	–	0.135	–	0.632	0.047	–	0.573	0.183	0.078	0.078	0.300
	2–12	0.440	0.228	0.000*	0.000*	0.495	–	0.270	–	0.301	0.007	–	0.615	0.248	0.947	0.000*	0.589
	6–12	0.984	0.736	–	0.000*	0.490	–	0.589	–	0.540	0.003*	–	0.949	0.081	0.000*	0.001*	0.589

[a] cfu: colony forming units/ml; TAc: total acids (mM); DH: degree of hydrolysis (%). C: concentration of enzyme (mg/kg$_{protein}$), T: time of hydrolysis (h).
* Significant at 5%.

Table 5
Parameter estimates in mathematical models encompassing microbial growth, amino acid formation, lactose consumption and protein hydrolysis fitted by non-linear regression to the experimental data[a]

Predictor variable	Dimensional form		Dimensionless	Protease 40 g/kg$_{protein}$		Protease 20 g/kg$_{protein}$		Trypsin 40 g/kg$_{protein}$		Trypsin 20 g/kg protein	
	Parameter	units	parameter	Dimensional	dimensionless	dimensional	dimensionless	dimensional	dimensionless	Dimensional	Dimensionless
Microbial growth											
	$C_{X,0}$	cfu/g	$C^*_{X,0}$	33.5	1.00×10^{-6}	29.5	1.00×10^{-6}	2.32	9.15×10^{-8}	3.65	9.15×10^{-8}
	$C_{A,1}$	g/l	$C^*_{A,1}$	0.58	6.65	0.28	3.14	0.15	1.79	0.10	0.995
	μ_{max}	h^{-1}	μ^*_{max}	0.49	0.49	0.29	0.29	1.59	1.59	1.41	1.41
	K_L	g/l	K^*_L	1.67	3.88×10^{-2}	1.72	4.00×10^{-2}	1.72×10^{-2}	4.00×10^{-4}	5.80×10^{-3}	1.22×10^{-4}
	K_A	g/l	K^*_A	8.70×10^{-6}	1.00×10^{-3}	8.70×10^{-6}	1.00×10^{-3}	8.50×10^{-6}	1.00×10^{-3}	9.08×10^{-6}	1.00×10^{-3}
Amino acid formation											
	v_{max}	g/(l h)	v^*_{max}	0.86	9.94	0.50	5.79	0.15	1.77	6.33×10^{-2}	0.74
	K_m	g/l	K^*_m	17.58	2.12	15.84	1.91	19.91	2.40	12.36	1.49
	Y_A	–	Y^*_A	1.10×10^{8}	0.29	3.41×10^{9}	10.01	3.09×10^{9}	10.40	3.26×10^{9}	10.80
Lactose consumption											
	Y_L	–	Y^*_L	1.22	52.70	1.22	52.50	0.14	6.03	0.13	5.45
Protein hydrolysis											
	α	–	α^*	4.22	4.40×10^{-2}	8.66	9.03×10^{-2}	35.69	0.37	70.97	0.78

[a] $C_{X,0}$: Estimate of initial viable numbers; $C_{A,1}$: Estimate of initial free amino acid concentration.

40 g/kg the estimates for v_{max} become approximately twofold. The value of K_m is similar for both enzymes and concentrations, as expected. The estimate for Y_A is poor and not prone to any realizable meaning. Interestingly, the estimate of α is much lower for Protease 2A (for which higher concentration of free amino acids is promoted), and when the concentration of either enzyme changes from 20 to 40 g/kg the value of α decreases to approximately one half.

4. Conclusions

Both enzymes tested bring about higher extents of hydrolysis when their concentrations are higher, as expected. Protease 2A produces a hydrolyzate richer in free amino acids, probably owing to its much lower specificity, whereas Trypsin produces a hydrolyzate richer in peptides. The differences in behavior, as time elapses, between Trypsin and Protease 2A hydrolyzates were significant in terms of all experimental parameters measured, except concentration of Glu and amount of peptide fraction produced from IgG and β-Lg. The growth of adventitious bacteria, and especially the formation of free amino acids, can thus be modeled using simple unstructured, unsegregated models.

Acknowledgements

Funding for author M. E. P. was provided by a Ph.D. fellowship issued within the framework of CIENCIA (BD-2526/93-IF), administered by JNICT, Portugal. Funding for author A. E. P. was provided by a Laboratory Technician fellowship issued within the framework of PRAXIS XXI (BTL-7506/96-IF), administered by JNICT, Portugal. Partial funding for this R&D work was obtained from project *Recuperação de proteínas e produção de goma alimentar a partir de soro lácteo* (PBICT/C/BIO1991/95), coordinated by F. X. M. and administered by JNICT, Portugal.

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